ANALYSIS OF CALRETICULIN (CALR) MUTATION IN MYELOFIBROSIS PATIENTS IN KHYBER PAKHTUNKHWA

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ABSTRACT

Objective: To analyze the prevalence of CALR and JAK2 mutation co-occurrence in myelofibrosis (MF) patients in KP.

Methods: This cross-sectional study included Myelofibrosis (MF) patients (n = 50) enrolled in the Hematology/Oncology department of Hayatabad Medical Complex (HMC), Institute of Radiotherapy and Nuclear Medicine, Peshawar (IRNUM) and Blood Diseases Clinic, Peshawar. Non-probability convenience sampling technique was used. All patients with JAK2 V617F positive and negative primary or secondary MF were included. After taking blood samples, DNA was extracted manually and analyzed for JAK2 mutations using conventional PCR. Sanger sequencing technique was employed to analyze the samples for CALR mutations. Data were recorded & analyzed statistically.

Results: Among the 50 patients, 48(96%) had JAK2 mutations. The typical CALR mutations was not identified in the current study. However, two specific genetic variants were identified in 30 patients i.e., a single nucleotide polymorphism (c.1381 G > T) in the 3' UTR and a novel insertion-deletion variant (c. 1099 CTT > AC). Seven patients had the Indel frameshift variant (p.Leu 367 Thr Fx 63), whereas 23 had SNP. In JAK-2 positive patients (n=28), six had the Indel variant, and 22 had the SNP. Two patients were JAK2 negative, one with SNP and the other with a frameshift mutation.

Conclusion: Majority of the MF patients had a novel frameshift variant (p. Leu367Thr Fx63) (n = 07) and an SNP in the 3' UTR (G > T) (n = 23) in CALR gene.

Keywords: Myelofibrosis, Calreticulin, JAK2.

This article may be cited as: Humayun S, Khan MTM, Hassan J, Ali M, Farooq G. Anaylsis of Calreticulin (CALR) mutation in Myelofibrosis patients in Khyber Pakhtunkhwa. J Med Sci 2022 ;30(1): 3-8

INTRODUCTION

Myelofibrosis (MF) is a clonal stem cell aberration that, like polycythemia vera (PV) and essential thrombocythemia, is categorized as a Philadelphia (Ph) chromosome-negative myeloproliferative neoplasm (MPN)[1] The disease is characterized by hepatosplenomegaly, cytopenia, thrombosis and fibrous bone marrow[2]. An yearly incidence of 0.5-1.5 cases per 100,000 people has made it the least prevalent myeloproliferative disease[3] MF often affects those over the age of 65, however, it can impact any age group. The median survival time is between 3.5 and 5.5 years. The most common cause of death includes infections, haemorrhage, thromboembolism, and development of acute leukemia[4].

Reasons behind the onset of myelofibrosis and other MPNs can be grouped into two: 1) phenotypic driver mutations and 2) sub-clonal or co-operating mutations. These mutations can increase and change the effects of phenotypic driving mutations in a variety of ways. Mutations in the thrombopoietin receptor gene, the Janus kinase 2 (JAK2) gene, and the calreticulin gene (CALR) are the three most often occurring phenotypic mutations in humans[5]. A small percentage of MPN patients have no mutation in any of these three genes, and is referred to as "triple-negative"[6]. Several studies have demonstrated that the absence of mutations in these genes is associated with poor prognosis when compared to subgroups with a mutation[7]. The JAK2 V617F mutation is found in exon 14 of the JAK2 gene, which is situated on chromosome 9p24, and affects majority of MF patients (50-60 %)[8]. There have been somatic mutations detected in the CALR gene, which is located on chromosome 19p13.2 found in

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Date Received: 16-12-2021
Date Revised: 02-03-2022
Date Accepted: 06-03-2022

35% of JAK2 mutation PMF patients[9]. However, multiple recent investigations have documented coexistence of CALR and JAK2 mutations in MPNs, which is contrary to conventional wisdom[10]. CALR is a chaperon protein found in the endoplasmic reticulum (ER), where it is critical for calcium homeostasis as well as the proper folding of newly generated glycoproteins[11]. It also plays an important part in the processes of cell proliferation, apoptosis, and immune-mediated cell death[12]. Three structural and functional domains of CALR protein are identified i.e., N, C, and P domains. The domain "C" is an acidic domain with a negative charge with many calcium-binding sites[13]. Because CALR mutations are the second most common cause of myelofibrosis, screening should be included in the diagnostic process for patients with myelofibrosis that is not caused by a genetic alteration in JAK2[14]. Based on the findings of multivariate regression analysis, overall survival was better in patients with CALR mutations than JAK2 or MPL mutations. Unselected myelofibrosis patients with CALR mutations account for 15.6-35% of all cases, compared to 56–88% of individuals with JAK2 or MPL negative myelofibrosis[15]. Co-occurrence of CALR and JAK2 mutations have a significant bearing on the clinical outcomes of myelofibrosis patients, accordingly, modifying treatment strategy[16]. The current study was designed to determine the frequency of JAK2 and CALR mutations in myelofibrosis patients in Khyber Pakhtunkhwa, Pakistan.

MATERIALS AND METHODS

A cross-sectional (descriptive) design was set for the study. The study was approved by the Ethical Committee, Khyber Medical University (KMU), Peshawar (Approval Number: DIR/KMU-EB/CM/000493). The research was carried out in the Department of Pathology, Institute of Basic Medical Sciences (IBMS), Khyber Medical University (KMU) Peshawar from June 2018 to May 2020. A total of 50 patients were enrolled at Hematology/Oncology department of Hayatabad Medical Complex (HMC), Institute of Radiotherapy and Nuclear Medicine, Peshawar (IRNUM) and Blood Diseases Clinic, Peshawar. The sample size was calculated using OpenEpi®. The margin of error used was 14%, the anticipated population proportion was 35% and the confidence interval was kept at 95% [17]. A non-probability sampling strategy was used. Patients with primary or secondary myelofibrosis due to ET & PV who tested positive for JAK2 V617F were included in the study. However, patients suffering from secondary MF because of other conditions such as tuberculosis, mycoses, lymphoma (Hodgkin or Non-Hodgkin), and other types of MPNs (PV, CML and ET) were excluded from the study. All of the patients provided informed and written consent before participating in the study. Information was gathered by using a specially designed proforma that included patients' demographic information, clinical history, physical examination, and investigations profile. Following standard protocols for blood sample transportation, blood samples were collected from the patients and carried to the IBMS-KMU, where they were processed at the hematology laboratory and stored until final analyses were performed. DNA was isolated from blood samples by a manual method of DNA extraction. For detection of JAK2 and CALR gene polymorphisms in diseased patients, allele-specific primers were designed. For JAK2 V617F mutation detection, conventional PCR was used. The PCR amplified product was confirmed on 1.5% agarose gel according to the established protocol. The gel was finally visualized on a UV transilluminator. For CALR sequencing, 50 patient's samples were amplified and sent to Macrogen, Inc.® in Seoul, South Korea. Sanger sequencing technology was used to detect CALR mutations in both JAK2 positive & negative patients. Statistical analyses were performed using IBM SPSS® (version 23) software. Simple arithmetic analyses (mean, standard deviation and/or percentages) were deduced for each parameter. Age and gender-wise stratification were done.

RESULTS

The study participants were between the ages of 20 and 80 years of age. Males comprised 34 (68%) of the 50 MF patients, while females comprised 16 (32%). There was a mean age of 50.60 ± 13.01 years in the study population. Male patients were 53.32±12.66 years old, while female patients were 44.81±12.36 years old. (Table 1). The mean haemoglobin, leukocytes and platelets count with minimum and maximum values are summarized in Table 2. Participants in the study were classified into three groups based on their hemoglobin level: (i) Severe anemia (Hb 6.5-7.9 g/dL), (ii) Moderate anemia (8 to 9.4gm/dL), and (iii) Mild anemia (9.5 to 11gm/dL). This classification was made using the World Health Organization (WHO) anemia grading system[18]. In the current study, 36 (72%) of the patients had anemia, whereas the remaining 14 (28%) had normal haemoglobin levels. Nineteen of the 36 individuals with anemia had severe anemia, 12 had moderate anemia, and 05 had mild anemia. Patients with thrombocytopenia were classified into four groups based on platelet count: Grade I Thrombocytopenia (platelets count 76-150 x 109 cells/L), Grade II Thrombocytopenia (platelets count 50-75 x 109 cells/L), Grade III Thrombocytopenia (platelets count 25-50 x 109 cells/L), and Grade IV Thrombocytopenia (platelets count < 25x109cells/L) [19]. The grade I thrombocytopenia was seen in 09 patients, Grade III in 03 patients, whereas 02 patients had Grade IV thrombocytopenia. Grade II Thrombocytopenia was not observed in any patients, while 03(6%) patients had a higher platelet level (> 450 x 109 cells/L.

In our study, 42 (82%) patients were identified as having primary myelofibrosis (PMF), whereas 09 (18%) had secondary MF due to PV 06 (12%) and ET 03 (06%). JAK2 mutations were found in 48 (96%) of the 50 patients with myelofibrosis, while it was not found in 2 (4%) pa-

tients. PCR amplified products are shown in Fig 1. Typical CALR mutations reported previously could not be identified in the current study upon sequencing. However, another novel variant and a single nucleotide polymorphism (SNP) were identified in a significant number of patients (n = 30, 60%), as shown in Table 3. Rest of the 20 patients did not have variant in the target gene. Novel Indel variant was found in seven patients, whereas 23 patients had the SNP in 3' UTR. The variant (p. Leu367Thr Fx 63) is a frameshift mutation with a forward shift of stop codon, where a substitution of CTT>AC was identified at position 1099 of the cDNA (c.1099 CTT>AC). The SNP identified in the current study was due to thymine substitution for guanine (ancestral nucleotide) at position 1381 of the cDNA (c.1381 G>T) in the 3' UTR region (Fig 2 and Fig 3). The identified genetic variations were also stratified with JAK2 mutation. Six patients with novel Indel variant were JAK2 positive while a single case was JAK2 negative. While, among the 23 patients with SNP, 22 were positive for a JAK2 mutation, whereas only one patient lacked the JAK2 mutation which was not statistically significant (p-value = 0.25).

Table 1: Age-wise Classification of Patients (n = 50)

Age Groups	Frequency	Percentage
Group I (20 to 40 yrs.)	13	26%
Group II (41 to 60 yrs.)	26	52%
Group III (61 to 80 yrs.)	11	22%

Table 2: Description of hematological parameters of patients (n = 50)

Parameters	Hemoglobin (g/dl)	Total leucocyte count (cells/L)	Platelet count (cells/L)
Mean	9.426 ± 3.08	19.5 x 109	272.64 x 109
Minimum	5.5	1.19 x 109	10 x 109
Maximum	18.8	77.60 x 109	656 x 109

Table 3: Genetic variations (SNP & Indel mutation) in the study population (n = 30)

Nucleotide Change	Outcome	Location	Nature of Variant	Variation No
c.1099 CTT > AC	p. Leu 367 Thr Fx 63		Indel Frameshift mutation	
c.1381 G > T	3' UTR Variant	1254+54-73	SNP	rs 1049481

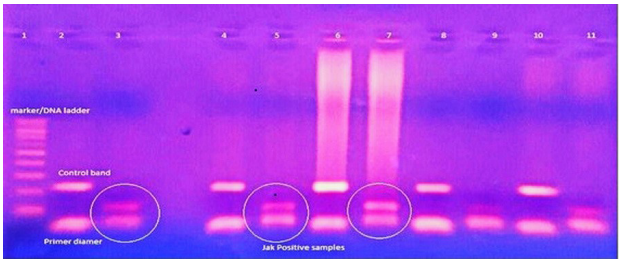

Fig 1. Gel Electrophoresis of JAK2 positive sample: (Left to Right) Lane 1: DNA gene ruler ladder. Lane 2: GG (Control), Lane 3: Protein dimers: Lane 4: GG (Control), Lane 5: TT. PCR product size of 460bp for Allele specific and Controls.

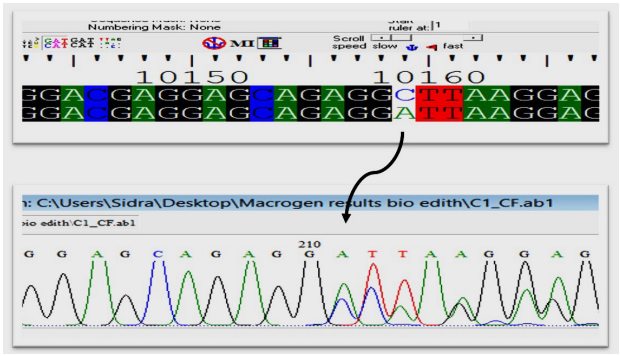

Fig 2: Chromatograph showing the CTT substitution by AC (CTT > AC)

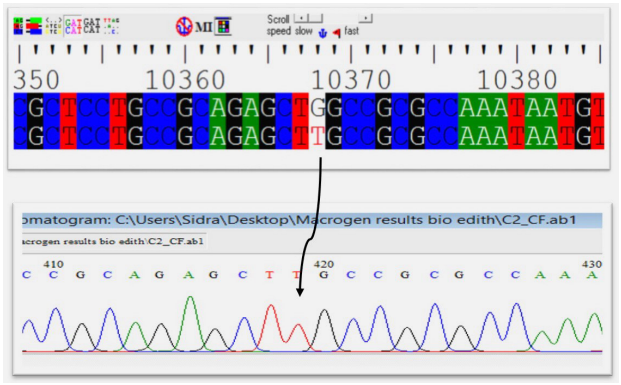

Fig 3: Chromatograph showing the G/T change in the SNP.

DISCUSSION

CALR mutations were thought to be mutually exclusive with MPL and JAK2 since their discovery in late 2013. A few recent studies have verified coexistence of the JAK2 V617F mutation with CALR mutations[14,20,21]. Our study was designed to assess the prevalence of mutations in the CALR gene in myelofibrosis patients with or without JAK2 mutations. The study included 50 patients with primary and secondary myelofibrosis. In this study, the majority of the patients (68%) were male, and the majority of them were between the ages of 41 and 60 (Age Group II), with a median age of 50. These findings are endorsed by

a study of 1000 MF patients in the United States, which found a male predominance (male: female = 3:2) and a high connection with old age (median age of 60 years)[22]. The greater median age may reflect the US population's overall longer life expectancy as compared to the Pakistani population. Another population-based investigation of 21 cases of agnogenic myeloid metaplasia (AMM) in Olmsted County, Minnesota (USA), revealed comparable findings of greater median age and male predominance[23]. Around 5-17% of people are diagnosed earlier than the ages of 40 and 50[24]. In our study, 26% of patients were under the age of 40, which is greater than in earlier studies. Previous studies have shown a significant frequency of anemia among MF patients pertaining to the prescribed treatment i.e., JAK2 inhibitors[25].

The JAK2 mutation was identified in 48(96%) patients, whereas only 02 (4%) patients did not carry this mutation. However, previously the frequency of JAK2 V617F mutation in myelofibrosis patients had been reported to be between 50-60%[26]. Patients are often misdiagnosed or diagnosed at a later stage of the disease due to the lack of facilities in the local healthcare system. Transformation of PV or ET has not been previously elucidated in the local population. The pathogenic role of CALR/JAK2 double mutations is still unknown, and it is unknown whether this will be another subgroup of MPNs in the future. In our study, JAK2/CALR double mutations were studied concerning primary and secondary myelofibrosis. The typical CALR mutations reported previously could not be identified in the current study. However, another novel variant and a single nucleotide polymorphism (SNP) were identified in a significant number of patients (n = 30, 60%). The novel Indel variant was found in 07 patients, 06 of whom were JAK2 positive, while a single case was JAK2 negative. A substitution of CTT>AC was identified at position 1099 of the cDNA (c.1099 CTT>AC). This variant (p. Leu367Thr Fx 63) is a frameshift mutation with a forward shift of stop codon. The integrity of the C-domain is imperative for the physiological functions of CALR protein. Damage to the C-domain is anticipated to culminate in loss of its function in CALR protein. Indel frameshift mutation of the kind identified in the current study is deemed to damage the protein structure at C-domain significantly. Future studies should be designed to determine this mutation and the resultant phenotypic effects on myelofibrosis patients.

The SNP identified in CALR gene in the current study was due to thymine substitution for guanine (ancestral nucleotide) at position 1381 of the cDNA (c.1381 G>T) in the 3' UTR region. The residue is located in the P-domain of the CALR protein which is found in the dbSNP (NCBI) database with a respective single-nucleotide number 1049481 (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1049481). These SNPs were identified in 23 patients; twenty two patients were JAK2 positive, while only one was JAK2 negative. The clinical and pathological significance of this SNP has not been reported yet. It is suggested that prognostic and therapeutic implications of this SNP should be studied in detail.

There are two limitations of our study. As the study consists of only 50 patients with myelofibrosis which was due to less prevalence of disease in our region, it is very early to make a conclusive decision on occurrence of JAK2/CALR double mutations. For this, a large patient's sample size is needed which may be studies in future studies. The other limitation of our study is that we did not measure the percentage of peripheral blood blasts i.e., studies showed that myelofibrosis patients with more than 4% peripheral blasts have poor prognosis.

CONCLUSION

Our study concluded that majority of the MF patients have JAK2 mutation. Most of the patients have a novel frameshift variant (p. Leu367Thr Fx63) and an SNP in the 3' UTR of the CALR gene. Large-scale studies are required to elucidate the pathogenicity of newly identified frameshift variant and the prognostic and therapeutic implications of the observed SNP.

ACKNOWLEDGEMENT

The authors acknowledge technical support provided by the staff at Department of Hematology, IBMS-KMU, Peshawar, Pakistan for technical support during the study.

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Author Declaration: The article is based on M.Phil (Hematology) research thesis of **Dr. Sidra Humayun**, Khyber Medical University Peshawar.

CONFLICT OF INTEREST: Authors declare no conflict of interest

GRANT SUPPORT AND FINANCIAL DISCLOSURE: NIL

AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under

Humayun S:	Conceived the idea, Literature review, Manuscript writing
Khan MTM:	Sample collection and Laboratory Work, Final approval of draft
Hassan J:	Sample collection, Bibliography
Ali M:	Laboratory work and Data analysis
Farooq G:	Results compilation and statistical analysis

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.